Accepted in Pest Management Science on 22 October 2019 <https://doi.org/10.1002/ps.5658>

Title: Effects of predator cues and pesticide resistance on the toxicity of a (bio)pesticide mixture

Short running title: Predators, resistance & mixture toxicity

Vienna Delnat^a, Lizanne Janssens^b and Robby Stoks^c

Evolutionary Stress Ecology and Ecotoxicology, University of Leuven, Belgium

^a vienna.delnat@kuleuven.be

^b lizanne.janssens@kuleuven.be

^c robbly.stoks@kuleuven.be

Corresponding author: Vienna Delnat, Charles Deberiotstraat 32, box 2439, 3000 Leuven, Belgium, tel. +32 16 37 65 27, vienna.delnat@kuleuven.be

Conflict of Interest: none

Abstract

BACKGROUND: Populations of target species are typically exposed to pesticide mixtures and natural stressors such as predator cues, and are increasingly developing resistance to single pesticides. Nevertheless, we have poor knowledge whether natural stressors and the presence of pesticide resistance shape mixture toxicity. We tested the single and combined effects of the pesticide chlorpyrifos and the biopesticide *Bacillus thuringiensis israelensis* (Bti) on the survival of the Southern house mosquito (*Culex quinquefasciatus*, Say) and whether these effects were magnified by synthetic predator cues of *Notonecta* water bugs and differed between a chlorpyrifos-resistant (Ace-1R) and non-resistant (S-Lab) strain.

RESULTS: Single exposure to Bti caused mortality in both strains (S-Lab ~27%, Ace-1R ~41%) and single exposure to chlorpyrifos caused only mortality in the S-Lab strain (~33%), while predator cues did not induce mortality. The chlorpyrifos-resistant strain was 1.5 fold more sensitive to Bti, indicating a cost of resistance. The interaction types between chlorpyrifos and Bti (additive), between chlorpyrifos and predator cues (additive), and between Bti and predator cues (synergistic) were consistent in both strains. Despite predator cues making Bti ~8% more lethal, they did not change the additive interaction between Bti and chlorpyrifos in their mixture in either strain.

CONCLUSION: These results indicate that the resistance against chlorpyrifos was not partly lifted when chlorpyrifos exposure was combined with Bti and predator cues. Identifying the interaction type within pesticide mixtures and how this depends on natural stressors is important to select control strategies that give a disadvantage to resistant individuals compared to non-resistant individuals.

Keywords: integrated pest management (IPM); mixture toxicity; pesticide resistance; semiochemicals; synergism; vector control

1. INTRODUCTION

Organisms are increasingly confronted with mixtures of pesticides generating increasing interest for their combined effects for control of target organisms.^{1,2} Pesticides may interact in three ways:^{3,4} (i) additive, if the effect of the mixture equals the sum of the effects of the individual pesticides, (ii) antagonistic, if the effect of the mixture is smaller than the additive effect, or (iii) synergistic, if the effect of the mixture is larger than the additive effect. While most attention is going to interactions between synthetic pesticides,^{3,5} interactions may also occur between synthetic pesticides and biopesticides (e.g.⁶). A biopesticide is a mass-produced agent for pest control that is manufactured from a living microorganism or a natural product.⁷

There is increasing interest for mixtures of (bio)pesticides in integrated vector and pest management since single pesticides are becoming less efficient because of the build-up of resistance.⁸⁻¹⁰ When the pesticides in these mixtures interact synergistically, extra potential is created to lower the dose of individual pesticides to reduce the ecological damage to non-target species.¹¹ As the number of populations of pest species resistant to single pesticides is increasing rapidly,^{10,12} it is important to study the consequences of using a pesticide mixture when resistance to a certain pesticide is already present in a population.¹³ This scenario has two possible opposite outcomes that have mainly been studied for mixtures of pesticides. On the one hand, organisms resistant to one pesticide can be less sensitive to a second pesticide due to cross-resistance (e.g.¹⁴⁻¹⁶). For example, strains of the plant hopper *Laodelphax striatellus* resistant to the organophosphate chlorpyrifos showed cross-resistance against the pyrethroid deltamethrin and the neonicotinoid imidacloprid due to the overexpression of Cytochrome P450 genes.¹⁷ On the other hand, resistant organisms might also be more sensitive to another pesticide due to a cost of resistance.¹⁸ Yet, this has been much less documented.¹⁹⁻²¹ One rare example is the finding that a pyrethroid-resistant amphipod

Hyalella azteca population showed a frequent tendency to be more sensitive to DDT, sodium chloride and copper (II) sulfate.²⁰ Intriguingly, some studies even documented that synergistic interactions between pesticides (e.g.^{22,23}), and between synthetic pesticides and biopesticides,^{24,25} may partially overcome the resistance against synthetic pesticides in mosquitoes.

A potentially complicating factor when assessing the biological impact of (bio)pesticide mixtures is that the effects of the single (bio)pesticide can be magnified by widespread natural stressors such as predator cues (e.g.^{26–29}). For example, both natural and synthetic predator cues of *Notonecta* water bugs increased the mortality of the mosquito *Culex pipiens* when combined with the biopesticide *Bacillus thuringiensis israelensis*.³⁰ A possible reason for this synergistic effect is the reduced food intake and food assimilation caused by exposure to the predator cues, limiting the amount of energy to cope with the (bio)pesticide.^{26,31–33} Yet, whether predator cues also magnify the effects of a (bio)pesticide mixture, hence change the interaction type within such mixtures, is unknown.

Here we studied the effects of the organophosphorous pesticide chlorpyrifos (CPF), the biopesticide *Bacillus thuringiensis israelensis* (Bti) and their mixture on the mortality of mosquito larvae. As specific research aims we tested whether synthetic predator cues magnified the effect of the single (bio)pesticides and their mixture, and whether these effects differed between an organophosphate-resistant and non-resistant mosquito strain. We thereby tested the two opposite hypotheses that the sensitivity to the biopesticide Bti could be (i) lower in resistant mosquitoes due to cross-resistance (e.g.^{16,17}) or (ii) higher due to a possible cost of resistance (e.g.^{18,34}). This will also inform whether resistance to the pesticide can be partially countered by combining chlorpyrifos with Bti and/or predator cues (e.g.^{24,25}).

The chosen (bio)pesticides, chlorpyrifos and Bti, were recommended for control of mosquito larvae by WHOPES³⁵ in 2017, with Bti being still recommended by WHO PQ³⁶ and

widely used for mosquito control in Europe (e.g.³⁷⁻³⁹). Chlorpyrifos is a model pesticide for the class of organophosphorous pesticides, of which temephos and pirimiphos-methyl are still recommended by WHO PQ.³⁶ Chlorpyrifos inhibits acetylcholinesterase,⁴⁰ which may cause muscle spasms and eventually death.⁴¹ Bti is a gram positive bacteria that forms spores with proteins that are very toxic to larvae of mosquitoes.⁴² These proteins cause mortality by disturbing the osmoregulatory mechanisms of the cell membrane in the intestinal gut.⁴² Because of the high specificity of Bti, it is assumed to have a low ecological impact⁴³ (but see ⁴⁴). We chose to work with the synthetic predator cues of *Notonecta* backswimmers, as these are important predators of mosquito larvae⁴² and they are one of the very few predator cues whose chemical composition has been identified: it is a mixture of the hydrocarbons n-tricosane and n-heneicosane.⁴⁵

As study species we chose the Southern house mosquito (*Culex quinquefasciatus*, Say 1823) which is the target of major control programs. This species is an important vector of viruses such as the West Nile virus⁴⁶ and the Saint Louis encephalitis virus,⁴⁷ which can cause large economic and social costs.⁴⁸ Key to address our questions is the availability of two well-studied strains with the same genetic background:⁴⁹ the non-resistant S-Lab strain and the Ace-1R strain resistant against organophosphates (e.g.^{34,50,51}). The Ace-1R strain is resistant due to a mutation (a single amino-acid substitution) in the allele that protects the enzyme acetylcholinesterase (AChE) from being disabled by organophosphates.⁵² Therefore, any phenotypic differences between these strains can be directly and causally linked to the resistance allele, hence to resistance (e.g.,^{34,49,51,53,54}).

2. MATERIALS AND METHODS

2.1. Study animals and rearing

Because the study organism is an invertebrate, no permits or animal care protocols were required for this experiment by the Animal Ethics Committee of the University of Leuven.

The two strains of *C. quinquefasciatus* were obtained from a continuous culture maintained at the University Montpellier II (France). The non-resistant S-Lab strain originated from San Joaquin Valley of California in 1950 and has served ever since as the insecticide susceptible reference strain.⁵⁵ Once in the laboratory in Leuven, the strains were kept for >10 generations prior to the experiment. Prior to the experiment, the chlorpyrifos resistance of the Ace-1R strain was confirmed. We tested survival of both strains using replicated vials each with 20 larvae in the solvent control (S-Lab: 6 vials, Ace-1R: 3 vials) and in an exposure treatment to 0.9 µg/L chlorpyrifos (S-Lab: 6 vials, Ace-1R: 5 vials). This showed high survival in the solvent control for both strains (mean survival $\pm$ 1 SE; S-Lab: 100.0% $\pm$ 0.0%, Ace-1R: 93.3% $\pm$ 4.8%), while at 0.9 µg/L chlorpyrifos all animals of the S-Lab strain died, but survival remained high in the Ace-1R strain (94.4% $\pm$ 0.0%). Adults were maintained in cages (30 $\times$ 30 $\times$ 30 cm) within a climate chamber (24 °C, 70% relative humidity, L:D 14:10). A 6% glucose solution (D-Glucose anhydrous; Sigma-Aldrich, St. Louis, Missouri, USA) was provided as food. Additionally, the adults were fed at least once a week with defibrinated sheep blood (Thermo Fisher Scientific) that was contained in a parafilm bag and warmed to 37 °C using a heating block. To start the experiment, multiple sets of ~130 larvae (obtained by pooling 2 egg clutches) were each reared in 2 L containers (18 $\times$ 13.3 $\times$ 12.1 cm). All 2 L containers were filled with aerated dechlorinated tap water (1 L) and placed in a temperature-controlled room (mean $\pm$ SE: 24.19 $\pm$ 0.03 °C, *N* = 1239, L:D 14:10). Larvae were fed three times a week with 3.5 mL of a 20 mg/mL mixture of Olvarit® 7 cereal flakes (46%), wheat germs (51%) and Supradyn® vitamins (3%).³⁰ This equals 0.300 mg of food/day/larva which is *ad libitum*.

2.2. Experimental design

The experiment had sixteen treatment combinations: 2 *C. quinquefasciatus* strains (S-Lab, Ace-1R) $\times$ 2 Bti treatments (absent, present) $\times$ 2 chlorpyrifos treatments (absent, present) $\times$ 2

predator cue treatments (absent, present). Larvae were exposed in groups of 20 in 200 mL glass vials for three days, starting within 24h after the moult into the final (L4) larval stage. The L4 stage is the most resistant larval stage and is recommended by WHO to test mosquito larvicides.⁵⁶ The medium was refreshed every 24h. The number of replicate vials per strain ranged from 7 to 20 (total of 160-400 mosquitoes) for each treatment combination (exact replicate numbers are shown in Figure 1). The variation in the number of replicates was due to extreme mortality after exposing animals after an error when replacing the chlorpyrifos stock solution; this caused us to discard all vials for which this stock solution was used. The number of vials that were used in the statistical analysis equalled 185 (matching 3,700 mosquitoes).

Mortality until the end of the three-day pesticide exposure period was checked daily. During this pesticide exposure period, no pupae were detected. Pre-trials showed that the first pupae occurred after six days at the given food level and rearing temperature of 24 °C. The amount of food was adjusted daily according to the number of surviving larvae to keep the food level constant per larva (0.300 mg/day/larva) and thereby avoid possible effects of density.

2.3. (Bio)pesticide and predator cue concentrations

The commercial Bti formulation VectoBac (WG, 37.4% Bti at 3000 ITU/mg, strain: AM 65-52) was purchased at Technic 3D (Paris, France). Chlorpyrifos (CPF), n-tricosane and n-heneicosane were purchased at Sigma-Aldrich (St. Louis, Missouri, USA; purity grade $\geq$ 99%).

To ensure that possible synergistic interactions on mortality could be studied, we selected concentrations of Bti and chlorpyrifos that individually induced approximately 30% mortality during a three-day exposure period ($LC_{30,72h}$). Based on a range finding experiment, this was 31 μ g/L for Bti and 0.22 μ g/L for chlorpyrifos. Both concentrations are ecologically relevant since these concentrations are equal or lower than the recommended dose to control

170 mosquito larvae by WHOPEs³⁵ guidelines which are 25-150 µg/L for Bti and 2.2-5 µg/L for
171 chlorpyrifos. These doses were based on a water depth of 0.5 m, matching the shallow water
172 bodies preferred by *Culex quinquefasciatus*.⁴² A stock solution (100 µg/mL) of chlorpyrifos
173 dissolved in ethanol absolute was used to prepare the chlorpyrifos solution (stored in the dark
174 at 4 °C). From this stock solution, a second stock solution (1 µg/mL) was made in Milli-Q
175 water. We took pooled water samples from three jars of the chlorpyrifos treatments at the start
176 and after 24h (before the next renewal of the medium). Chlorpyrifos samples were analysed at
177 the Division of Soil and Water Management at the KU Leuven using UPLC-MS/MS with
178 Triple Quadrupole Mass Spectrometry. The initial chlorpyrifos concentration was 0.217
179 ± 0.058 µg/L (mean ± SE, *N* = 2). After 24 hours, the chlorpyrifos concentration was 0.066 ±
180 0.009 µg/L (*N* = 2). A daily stock solution (1 mg/mL) of Vectobac dissolved in Milli-Q water
181 was used to prepare the Bti solution. A second stock solution (100 µg/mL) was made in
182 Milli-Q water from this first stock solution.

183   The medium with synthetic predator cues of the backswimmer *Notonecta maculata*
184 was prepared following the protocol of Op de Beeck et al.³⁰ The medium with synthetic
185 predator cues was composed of n-tricosane (200 ng/L) and n-heneicosane (33 ng/L) dissolved
186 in dechlorinated tap water. The used concentrations of n-tricosane and n-heneicosane matched
187 those of a realistic density of 30 *Notonecta* individuals/L (based on ⁴⁵). A stock solution (1
188 mg/mL) was made for both hydrocarbons by dissolving granules in ethanol absolute (100%)
189 (Sigma-Aldrich, St. Louis, Missouri, USA). A secondary stock solution in Milli-Q water was
190 prepared from these primary stock solutions to obtain a n-tricosane solution (1 µg/mL) and a
191 n-heneicosane solution (0.1 µg/mL).

192   All solutions (including the Bti solution) contained 4.53 µL ethanol/L. This was
193 ca. 22 times below the maximum allowed solvent concentration recommended by the OECD

(100 µL/L)⁵⁷ and ca. 2.7 times lower than the lowest reported NOEC for aquatic invertebrates (9.6 mg/L).⁵⁸ We therefore only used a solvent control.

2.4. Statistical analyses

All statistical analyses were performed in R v3.3.2⁵⁹ with the packages lme4 v1.1-13⁶⁰, car v2.1-5⁶¹, afex v0.18-0⁶² and lsmeans v2.26-3.⁶³

Effects of the mosquito strain, exposure to chlorpyrifos, exposure to Bti and exposure to the predator cues on mortality (0 = alive, 1 = dead) after the three-day pesticide exposure period were analysed using a generalized linear mixed model with a binomial error structure and the logit link. Besides these four treatment factors, the model also included all possible two- to four-way interactions between these factors. The model fitted our data well as indicated by a dispersion parameter of 1.004. Because sets of 20 larvae were exposed simultaneously within one vial, vial was added as random factor to avoid pseudoreplication. Because of the unbalanced number of replicated vials per treatment combination, we used Type III Sums of squares.^{64,65} When interaction effects between Bti, chlorpyrifos, predator cues and/or strain were detected, we used Tukey HSD post hoc tests to identify which treatment combinations differed.

It is important to note that linear models use an additive null model, hence cannot be used to identify the interaction type between stressors when both are lethal. Indeed, linear models may falsely identify antagonistic interaction effects between two lethal stressors as they do not account for the probability that an individual cannot be killed by two stressors simultaneously (Figure 1 in Côté et al.⁶⁶).⁶⁷ To test for interactions between stressor combinations that included the two lethal stressors (here Bti and chlorpyrifos) we therefore identified the interaction type with the independent action model. This model is further advocated when both chemicals have a different mode of action.⁶⁷⁻⁶⁹ We determined this interaction type per strain given that organophosphate-resistance may change the interaction

type. We applied the model following the procedure detailed in Coors and De Meester.⁷⁰ First, the observed absolute effects of the single pesticides were expressed as proportional effects with the equation $E_i = \frac{(e_i - e_{control})}{(e_{max} - e_{control})}$. Hereby, e_i is the single-pesticide effect in percentage, $e_{control}$ is the effect of the pesticide-free control in percentage, and e_{max} is the maximum possible effect of that single-pesticide which is 100 for mortality. Because single exposure to predator cues had no effect on mortality in any strain (see results), $e_{predator\ cues}$ was equal to $e_{control}$. Second, the predicted joint effects were calculated using the equation $E_{mix} = 1 - \prod_i (1 - E_i)$. Third, the predicted joint effect E_{mix} was back-transformed to percentage to allow comparison with the observed joint effects using the equation $e_{mix} = E_{mix} \times (e_{max} - e_{control}) + e_{control}$. We interpreted an observed effect being significantly different from the predicted joint effect by the model if the predicted effect was not within the 95% confidence interval of the observed effect in percentage. A significantly weaker observed joint effect compared to the predicted joint effect indicates an antagonistic interaction, while a significantly stronger observed joint effect indicates a synergistic interaction. To specifically address whether predator cues had an effect on the interaction type in the two-component mixture, we separately applied independent action models in the absence and in the presence of predator cues (for details of the approach, see Supporting Information). Note that these independent action models for the two-component model (also in the presence of predator cues) are not the same as the independent action model used to test the interaction effect of the three-component model (see Supporting Information).

3. RESULTS

3.1. Strain-specific effects of the (bio)pesticides

The single effects of both pesticides differed between strains (Strain $\times$ Bti and Strain $\times$ CPF, Table 1, Figure 1). Single exposure to Bti increased larval mortality by the end of the three-day exposure period in both strains (Tukey: both p -values < 0.001), yet this lethal effect was

stronger in the resistant Ace-1R strain (41%) than in the non-resistant S-Lab strain (27%) (Strain $\times$ Bti, Table 1, Figure 1). Single exposure to chlorpyrifos only increased mortality in the non-resistant S-Lab strain with ~33% (Tukey: $p < 0.001$) and not in the resistant Ace-1R strain (Tukey: $p = 0.33$) (Strain $\times$ CPF, Table 1, Figure 1). Furthermore, also the Strain $\times$ Bti $\times$ CPF interaction was significant (Table 1), indicating that the difference in mortality between both strains depended on the combination of Bti and chlorpyrifos exposure. While the resistant Ace-1R strain had a higher mortality when exposed to Bti and a lower mortality when exposed to chlorpyrifos compared to the non-resistant S-Lab strain (Tukey: all p -values < 0.001), both strains did not differ in the control treatment or when exposed to the Bti-chlorpyrifos mixture (Tukey: both p -values ≥ 0.47).

3.2. Effect of predator cues on single (bio)pesticides

Exposure to predator cues did not have a main effect on larval mortality in either strain (Tukey: both p -values ≥ 0.43 , Fig. 1, Table 1). The two-way interaction between chlorpyrifos and predator cues was not significant, indicating an additive effect between both stressors (CPF $\times$ Cues, Fig. 1, Table 1). In contrast, the two-way interaction between Bti and predator cues was significant (Bti $\times$ Cues, Fig. 1, Table 1). While the predator cues were not lethal in isolation (Tukey: $p = 0.15$), they did increase the mortality in Bti-exposed larvae with ca. 8% compared to exposure to Bti alone ($p = 0.040$). This indicates a synergism where the predator cues magnified the toxicity of Bti.

3.3. Effects of strain and predator cues on the interaction type of the (bio)pesticide mixture

The independent action model identified an additive effect between Bti and chlorpyrifos both for the non-resistant S-Lab strain and for the resistant Ace-1R strain in the absence of predator cues (Table 2A). To specifically address whether predator cues had an effect on the interaction type in the two-component mixture, a separate independent action model was run.

This model showed that for both strains, the interaction type between Bti and chlorpyrifos was also additive in the presence of synthetic predator cues (Table 2B). Thus, predator cues did not change the interaction type in the Bti-chlorpyrifos mixture. Also for the three-component mixture of Bti, chlorpyrifos and predator cues, the independent action model identified an additive effect in both strains (Table 2C).

4. DISCUSSION

4.1. Effects of single exposure to (bio)pesticides and predator cues

As expected, the organophosphate resistant Ace-1R strain was not affected when exposed to chlorpyrifos (0.22 µg/L) while the non-resistant strain suffered a higher mortality. There was no mortality difference between the resistant and the non-resistant mosquito strains in the control treatment. This contrasts with the general idea that resistant strains have a disadvantage in the absence of the pesticide due to the cost of resistance.¹⁸ For example, in the here studied resistant Ace-1R strain of *C. quinquefasciatus* costs have been observed in the absence of pesticides in terms of increased probability of being killed by predators³⁴ and reduced mating success.⁵¹ Similar to the current results, these studies did not report a higher mortality in the Ace-1R strain compared to the S-Lab strain in the absence of pesticides or natural enemies.^{34,51} This suggests that costs of resistance may be sublethal and too subtle to detect under optimal laboratory conditions.⁷¹ This lack of observable costs of resistance in the absence of the pesticide has been observed in other insects, e.g. when comparing a Bt Cry1Ac-resistant and non-resistant strain of the lepidopteran *Plutella xylostella*.⁷²

Resistant mosquitoes were more sensitive to the biopesticide Bti, but not to the predator cues. While lethal effects of exposure to predator cues have been observed in aquatic insects^{73,74} and the Ace-1R strain is more vulnerable to predation by aquatic bugs,³⁴ this did not translate into a higher vulnerability to the synthetic predator cues in our study. Instead, we did find a cost of resistance to chlorpyrifos in terms of an increased vulnerability of the Ace-

1R strain to the biopesticide Bti. Thus, the cost of pesticide resistance was only visible when exposed to another pesticide. Notably, most studies documented cross-resistance where resistance to one pesticide caused resistance to another pesticide (e.g.^{14,15,17,75}). Only three other studies documented that resistance against one pesticide caused an increased vulnerability against another pesticide.^{19–21} For example, the indoxacarb-resistant cotton bollworm *Helicoverpa armigera* was more susceptible to abamectin and methoxyfenozide.¹⁹ Such costs have been explained by two mechanisms depending on how resistance is realized.¹⁸ Firstly, when resistance is due to overexpression of detoxification genes, this is energetically costly, resulting in less energy allocation toward mechanisms to cope with a second pesticide. Secondly, when resistance is due to target-site modification, this may cause a constitutive low-expression of certain genes which can be essential for the viability of the organism. The latter may explain the observed cost in our study as the Ace-1R strain has a target-site modification that decreases the sensitivity to the pesticide at its site of action.⁵²

4.2. Effects of predator cues on the toxicity of single pesticides

Synthetic predator cues did not have a main effect on larval mortality, yet they magnified the lethal effect of Bti to the same extent in both strains. This confirms the synergistic interaction between Bti and synthetic predator cues of *Notonecta* sp. in the related Northern house mosquito (*Culex pipiens*).³⁰ Synergistic interactions between pesticides and environmental stressors can be expected when exposure to each individual factor is energetically costly.³¹

In contrast, the predator cues did not magnify the toxicity of chlorpyrifos in any strain. Although synergisms between synthetic pesticides and predator cues have often been observed (e.g.^{26–29,76}), these are not general (e.g.^{32,77}). Differences in interaction types between stressors are poorly understood,⁶⁶ but can possibly be due to differences in synthetic pesticide bioavailability and exposure concentration,³² hence differential energetic costs to cope with the synthetic pesticides.³¹

4.3. Interaction effects in the (bio)pesticide mixture

The independent action model identified the interaction type between chlorpyrifos and Bti to be additive. This corresponds with the additive effect between the organophosphate malathion and Bti for mortality in *Culex pipiens*.⁶ Yet, other studies found synergistic interactions between organophosphates and biopesticides.^{78,79} Again, as synergistic effects may be explained by the addition of energetic costs,³¹ differences in energy budgets when animals are exposed to different mixtures may be key to understand the presence of such synergisms. Nevertheless, our results march the emerging view that the most common interaction type in pesticide mixtures is an additive effect.^{3,80-82}

The interaction type between chlorpyrifos and Bti in their mixture did not differ between the non-resistant and the resistant strain. This contrasts with studies showing different interaction types in a binary pesticide mixture between strains resistant and non-resistant to one of the pesticides.^{22,23,83,84} For example, while both carbofuran and carbosulfan (carbamates) combined additively with acrinathrin (pyrethroid) in a susceptible strain, they interacted synergistically with acrinathrin in an acrinathrin-resistant field strain of the Western flower thrips *Frankliniella occidentalis* where resistance is caused by overexpression of monooxygenases.²² This synergism was explained by both carbamates inhibiting monooxygenases that were involved in metabolic detoxification of pyrethroids.²² This supports the general mechanism where a pesticide A inhibits a detoxification enzyme to cope with pesticide B and thus increases the toxicity of pesticide B when the resistance against B is caused by overexpression of that detoxification enzyme.⁸⁵ However, this general mechanism may not play in the Bti-chlorpyrifos mixture, because Bti does not have a known effect on the detoxification enzymes of chlorpyrifos.^{86,87} Moreover, the resistance against chlorpyrifos in the Ace-1R strain is caused by target-site modification and not by overexpression of detoxification enzymes.⁵²

While many studies have demonstrated that exposure to predator cues may increase the toxicity of single pesticides (e.g.^{26–29,76}), it has never been tested whether this also extends to pesticide mixtures. While predator cues did magnify the toxicity of Bti, this did not translate in exposure to predator cues changing the interaction type between chlorpyrifos and Bti. Furthermore, also the interaction type in the three-component mixture with Bti, chlorpyrifos and the synthetic predator cues was additive and did not differ between both strains. Since predator cues do not always affect the toxicity of single pesticides (e.g.^{32,77}, this study), more studies are needed to see if there is a general pattern for mixtures or not. Our results also indicate that the resistant strain did not become more sensitive to chlorpyrifos after co-exposure with Bti or predator cues. This contrasts with studies showing that some biopesticides are able to reduce or even alleviate the resistance to single synthetic pesticides in mosquitoes (e.g.^{24,25}).

5. CONCLUSION

How stressors interact is crucial for the control of pest populations.^{1,2,66,88} We here provided evidence that resistance to the pesticide chlorpyrifos did not affect the interaction types between the pesticide chlorpyrifos and the biopesticide Bti (additive), between chlorpyrifos and predator cues (additive), and between Bti and predator cues (synergistic). This was true despite the resistant strain being more sensitive to Bti. Also, the interaction type in the mixture with all three components was additive for both the non-resistant S-Lab strain and the resistant Ace-1R strain. This shows that the resistance against chlorpyrifos could not be partly lifted when chlorpyrifos exposure was combined with Bti and/or predator cues. Despite that both strains were differentially vulnerable to the (bio)pesticides, they showed the same mortality in the pesticide mixture. Furthermore, we provided the first experimental test whether predator cues, that are widespread in natural water bodies, can change the interaction type in a pesticide mixture. Despite the predator cues magnifying the toxicity of Bti, they did

not magnify the toxicity of the mixture. These findings are relevant for integrated pest management where target species are typically exposed to pesticide mixtures^{89,90} and natural stressors such as predator cues, and are increasingly developing resistance to single synthetic pesticides.^{10,12} Further studies are needed that test whether natural stressors such as predator cues magnify the toxicity of mixtures in pest species. Identifying the interaction type within pesticide mixtures and how this depends on natural stressors is important to select control strategies that give a disadvantage to resistant individuals compared to non-resistant individuals.

6. ACKNOWLEDGEMENTS

We thank the ESEE research group, especially Kiani Cuypers and Ana Elisabete Pires De Lima for their support and assistance during the experiment. We are very thankful to Prof. Dr. Mylène Weill for providing the mosquito strains. Dr. Anja Coors was of great help when applying the independent action model. Three anonymous reviewers provided constructive comments. VD is a PhD fellow of the Fund for Scientific Research Flanders (FWO). Financial support came from FWO research grant G.0943.15 and the KU Leuven grant C16/17/002.

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8. TABLES

Table 1. Results of the generalized linear mixed model testing for the effects of strain and exposure to *Bacillus thuringiensis israelensis* (Bti), chlorpyrifos (CPF) and predator cues (Cues) on the mortality of the mosquito *Culex quinquefasciatus*. The p -values indicated in bold are significant ($p \leq 0.05$).

	Mortality		
	χ^2	df	P
Strain	7.38	1	0.007
Bti	363.04	1	< 0.001
CPF	66.40	1	< 0.001
Cues	0.47	1	0.49
Strain $\times$ Bti	27.97	1	< 0.001
Strain $\times$ CPF	43.48	1	< 0.001
Strain $\times$ Cues	0.87	1	0.35
Bti $\times$ CPF	18.95	1	< 0.001
Bti $\times$ Cues	4.19	1	0.041
CPF $\times$ Cues	0.53	1	0.47
Strain $\times$ Bti $\times$ CPF	15.12	1	< 0.001
Strain $\times$ Bti $\times$ Cues	1.93	1	0.16
Strain $\times$ CPF $\times$ Cues	0.57	1	0.45
Bti $\times$ CPF $\times$ Cues	0.46	1	0.50
Strain $\times$ Bti $\times$ CPF $\times$ Cues	0.16	1	0.69

Table 2. Results of the independent action model identifying the interaction type for the non-resistant S-Lab strain and the resistant Ace-1R between (A) *Bacillus thuringiensis israelensis* (Bti) and chlorpyrifos (CPF) in their two-component mixture in the absence of predator cues, (B) Bti and chlorpyrifos in their two-component mixture in the presence of predator cues, (C) Bti, chlorpyrifos and predator cues in their three-component mixture. If the predicted value is within the range of the 95% confidence interval (CI) of the observed value, the interaction type is additive.

	Predicted (%)	Observed (%)	95% CI Observed	Interaction type
(A) two-component mixture Bti × CPF in the absence of predator cues				
S-Lab	52.27	50.71	[36.62, 64.81]	additive
Ace-1R	44.25	51.00	[37.47, 64.53]	additive
(B) two-component mixture Bti × CPF in the presence of predator cues				
S-Lab	55.80	55.00	[40.18, 69.82]	additive
Ace-1R	53.02	59.50	[43.94, 75.06]	additive
(C) three-component mixture Bti × CPF × predator cues				
S-Lab	52.27	55.00	[40.18, 69.82]	additive
Ace-1R	44.25	59.50	[43.94, 75.06]	additive

9. FIGURE LEGENDS

Figure 1. Mortality of the larvae of the non-resistant (S-Lab, light grey) and the resistant (Ace-1R, dark grey) strains of the mosquito *C. quinquefasciatus* in response to single and combined exposure to the synthetic pesticides chlorpyrifos (CPF), *Bacillus thuringiensis israelensis* (Bti) and synthetic predator cues (Cues). Mortality is expressed for each strain as the average percentage of all vials in that given treatment (± 1 SE). Numbers inside or just above the bars indicate the number of replicate vials (each vial contained 20 L4 larvae). Significance levels of Tukey tests comparing the mean mortality between both strains per treatment combinations are indicated above the bar plots (n.s.: $p > 0.05$, ***: $p \leq 0.001$).